

Recovery of IgG from ethanol precipitates of the Cohn-Oncley and Kistler-Nitschmann fractionation schemes

John Curling, Dev Baines, Crystal Russell, Keith Watson, Emma Ward, Hannah Pollard & Steve Burton
ProMetic BioSciences Ltd., 211 Cambridge Science Park, Cambridge CB4 0ZA, UK.

» Abstract

Current goals in IVIG manufacturing are to improve yield (output) while maintaining product quality. From a processing and licensing viewpoint the trunk fractionation scheme should not be changed. We have thus focused our efforts on the recovery of IgG from existing ethanol precipitates. Yield improvements can be achieved by reducing the number of steps through to the final product, by increasing individual step yields and by an integrated processing approach, eliminating "inter-step" adjustments.

Different ethanol precipitates were subject to conditioning steps to provide stable load solutions for chromatography. Fraction II+III paste was, e.g., re-suspended in acetate/phosphate buffer at pH 3.8 and held overnight, centrifuged, adjusted to pH 7, re-centrifuged and filtered. These conditions are indicative of a generalised approach of low pH extraction with neutral pH clarification. The II + III extract was loaded at a dynamic binding capacity of 18 g/L adsorbent onto MAbSorbent® A2P at neutral pH and eluted at low pH (3-3.5). Residual IgA was removed in a flow through mode using an anion exchanger, MAbSorbent A2P is a totally synthetic, non-proteinaceous, non-cell derived, triazinyl derivative immobilised on the 6% cross-linked agarose, PuraBead®.

An investigation was carried out to elucidate conditions for IgG elution at high pH. Optimal IgG recovery with the lowest IgA/IgM impurity levels was determined at pH 6 using a citrate-phosphate buffer with the addition of 4% polyethylene glycol (10 kDa). These elution schedules allow for a choice of low or high pH IgG elution.

» Methods

1. Preparation of precipitate extracts for chromatography

Simple extraction procedures employing sodium acetate + sodium phosphate buffers at low pH (pH 3.8 – 4.8), supplemented with sodium chloride are used to extract pastes from Cohn or Kistler-Nitschmann procedures. Extractions are performed overnight at 4°C. The insoluble material is removed by centrifugation, the extract adjusted to pH 7.0 and centrifuged after a further short hold. The extract is filtered at 1µm prior to adjustment to starting buffer conditions. These conditions are applicable to:

- Precipitate A
- Precipitate A+I
- Fraction II+III
- Fraction I+II+III

2. Chromatography on MAbSorbent® A2P

MAbSorbent A2P was packed to either 50 mL, 100mL or 300 mL column volumes and run at flow rates up to 200 cm/hr in all buffers at 20°C. The diameter to height ratio of the columns was generally 1:6 with bed heights of ca. 10 cm.

Columns were loaded with extract to a capacity equivalent to 21 grams/litre IgG. Columns were used in a binding mode and the IgG was collected in the elution fraction of ca. 4CV. No further elution buffer was used and the columns were subject to direct regeneration and sanitisation in 0.5 M sodium hydroxide prior to re-equilibration with 5CV starting buffer.

Starting/Equilibration buffer: 50mM sodium phosphate, 150mM sodium chloride, pH 7.0, 3CV
Elution buffer: 200 mM sodium acetate, 4% PEG, pH 6.0, 5CV.
 The concentration and molecular weight of the PEG were investigated.
Sanitisation (and removal of remaining protein): 0.5 M sodium hydroxide.

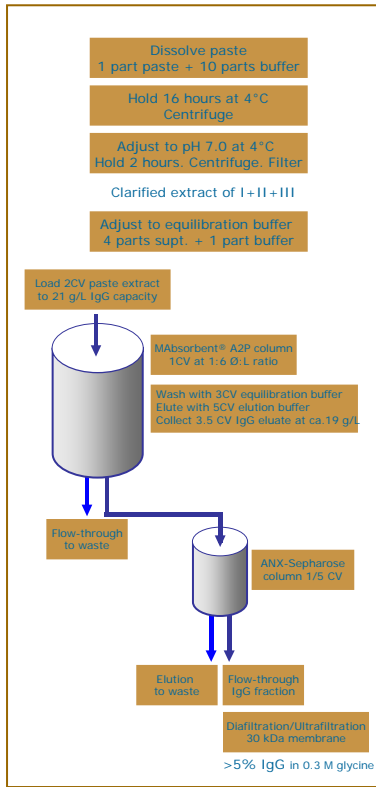
3. Chromatography on ANX-Sepharose®

ANX-Sepharose was packed into small columns, generally at 1/5 volume of the MAbSorbent column and equilibrated at pH 6, the elution pH from the previous step. The elution fraction from the MAbSorbent adsorption step was loaded directly on to the ANX-Sepharose without pH, ionic strength or volume adjustment. The load volume was the complete elution volume from the previous column. The column was used in a flow-through mode and the IgG fraction collected in ca. 5CV.

Starting/Equilibration buffer: 50mM sodium acetate, pH 6.0, 3CV
Elution buffer: 50mM sodium acetate, 1M sodium chloride, pH 6.0, 5CV.

The column was regenerated using equilibration buffer, 3CV.

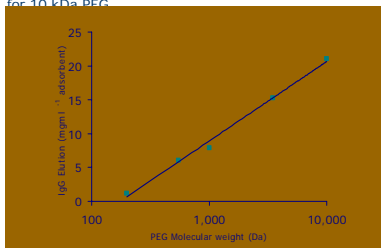
» Flow sheet



» Results

1. PEG molecular weight and elution pH

Low pH buffers, for example 10 mM sodium citrate, pH 3.0 or pH 3.5 are frequently used to elute antibodies from MAbSorbents. In this investigation the effect of addition of 2% w/v PEG (10 kDa) in sodium acetate and citrate buffers showed that 200 mM sodium acetate was adequate to effect higher pH elution. In addition, a direct relationship was found between the PEG molecular weight and elution enhancement indicating preference for 10 kDa PEG.



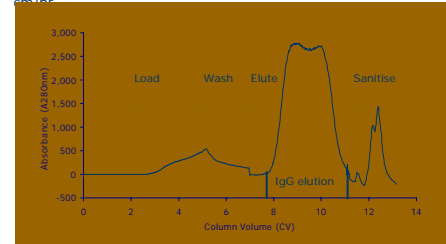
Effect of PEG molecular weight. 1% (w/v) solutions of PEG in a 50mM sodium phosphate buffer (pH 7.0) with 150mM sodium chloride.

2. Capacity, purity and flow rate of MAbSorbent A2P

In a flow rate vs. capacity vs. purity study columns were run at 45 cm/hr, 100 cm/hr and 200 cm/hr. Binding capacity drops from 25 g/L adsorbent at 45 cm/hr to 15.5 g/L at 200 cm/hr. Purity was maintained independent of flow rate at 92% with a reduction in IgA from 0.11 mg/mg IgG to 0.07-0.08 mg/mg IgG. IgM was cleared in all three cases. IgA, IgM, IgG were measured by nephelometry. Recovery dropped with increase of flow rate from 92% at 45 and 100 cm/hr to 85% at 200 cm/hr.

3. Elution profile over MAbSorbent A2P

The figure shows the elution profile of a I+II+III paste extract at the 300 mL column (5 cm Ø x 13.5 cm) scale run at 100 cm/hr.



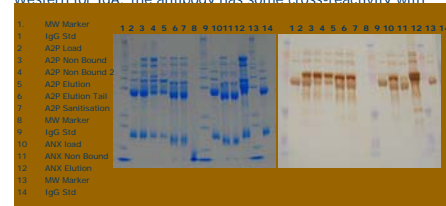
4. Removal of impurities

The table below shows the reduction of plasma protein impurities over the two column steps. All proteins were measured by nephelometry. Note that fibrinogen and plasminogen are removed in the extraction procedure. Fib = Fibrinogen, Pls = Plasminogen, Cer = Ceruloplasmin, Trf = Transferrin.

Extract	Total IgG	Total IgA	Total IgM	Fib	Pls	Cer	Trf	Wash	Eluate
Extract	17,500	2,085	768	0.12	0.04	0	0	0.11	0.01
A2P load	5,741	656	239	0.11	0.04	0	0	0.10	0.01
A2P elution	5,087	536	0	0.11	0.00	0	0	0.10	0.01
ANX load	5,092	493	0	0.10	0.00	0	0	0.11	0.01
ANX elution	4,954	0	0	0.00	0.00	0	0	0.05	0.01

5. SDS PAGE Electrophoresis and Western Blot (Anti-IgA)

The figure below shows SDS PAGE (reducing conditions) and a Western for IgA: the antibody has some cross-reactivity with

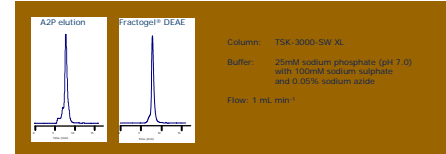


6. IgG sub-class distribution

The relative ratio of IgG sub-classes, from the purification of a I+III extract is shown below. Determinations by nephelometry.

Sub-class	IgG ₁	IgG ₂	IgG ₃	IgG ₄
I+III paste extract	64.1%	29.2%	2.4%	3.7%
MAbSorbent A2P elution	62.8%	29.2%	0.1%	5.0%

7. Purity by size exclusion HPLC



» Conclusions

- » Yield of IgG from Cohn and K-N pastes is 85 - 92%
- » Purity after a single MAbSorbent A2P step is 92%
- » The product can be polished using ANX-Sepharose
- » High pH elution at pH 6 using PEG (10kDa) is effective
- » Plasma sub-class distribution is conserved
- » IgG is free of IgA and IgM by nephelometry

» Acknowledgements

We are grateful to Baxter Biosciences (Glendale), USA and the Bio Products Laboratory, UK, for providing IgG containing pastes. Work on Precipitate A was done in collaboration with ZLB Bioplasma, Switzerland.

